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The effect of inoculum on the performance of sulfate-reducing columns treating heavy metal contaminated water

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ARTICLE INFO

Article history:

Received 27 April 2006

Received in revised form

8 November 2006

Accepted 10 November 2006

Available online 12 January 2007

Keywords:

Mine drainage treatment

Sulfate-reducing bacteria

Microbial ecology

Molecular biology

Environmental biotechnology

ABSTRACT

Sulfate-reducing permeable reactive zones (SR-PRZs) are a passive means of immobilizing metals and neutralizing the pH of mine drainage through microbially mediated reactions. In this bench-scale study, the influence of inoculum on the performance of columns simulating SR-PRZs was investigated using chemical and biomolecular analyses. Columns inoculated from two sources (bovine dairy manure (DM) and a previous sulfate-reducing column (SRC)) and uninoculated columns (U) were fed a simulated mine drainage and compared on the basis of pH neutralization and removal of cadmium, zinc, iron, and sulfate. Cadmium, zinc, and sulfate removal was significantly higher in SRC columns than in the DM and U columns, while there was no significant difference between the DM and U columns. Denaturing gradient gel electrophoresis (DGGE) analysis revealed differences in the microbial community composition among columns with different inocula, and indicated that the microbial community in the SRC columns was the first to reach a pseudo-steady state. In the SRC columns, a higher proportion of the DGGE band DNA sequences were related to microorganisms that carry out cellulose degradation, the rate-limiting step in SR-PRZ energy flow, than was the case in the other columns. The proportion of sulfate-reducing bacteria of the genus *Desulfobacterium* was monitored using real-time quantitative PCR and was observed to be consistently higher in the SRC columns. The results of this study suggest that the inoculum plays an important role in SR-PRZ performance. This is the first report providing a detailed analysis of the effect of different microbial inocula on the remediation of acid mine drainage.

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1. Introduction

The waters emanating from mine sites are typically characterized by low pH, high sulfate concentrations, and the presence of toxic heavy metals. Although some mine drainage issues are associated with large urban areas, many sites are located in remote areas and are subject to extreme weather conditions, which makes remediation challenging.

This is particularly true in the case of abandoned mine land (AML) sites and affected streams. Researchers have estimated that there are over 200,000 AML sites and 5000 to 10,000 miles of impacted streams scattered throughout the US (EPA, 1997).

There are two general approaches for remediating mining-influenced waters: active or passive. Active treatments (chemical treatment) require continuous inputs of resources

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doi:10.1016/j.watres.2006.11.025

to sustain the process, whereas passive treatments (biological treatment or anoxic limestone drains) require minimal inputs of resources once in operation (Johnson and Hallberg, 2005). Passive methods are preferred due to their low cost, low required maintenance, and because they produce minimal hazardous waste requiring disposal. In particular, sulfate-reducing permeable reactive zones (SR-PRZs), such as wetlands and subsurface reactive zones, are an attractive passive means of treating mine drainage. SR-PRZs contain solid organic substrates that support the growth of anaerobic microbial communities, including sulfate-reducing bacteria (SRB), which reduce sulfate in the mine drainage to sulfide. The sulfide in turn reacts with the heavy metals and immobilizes them within the zone as solid precipitates. SR-PRZs are typically inoculated with microbial communities from animal manure.

While there are many desirable aspects of SR-PRZs, there is little guidance available regarding their design. For this reason, it is not known why some have been observed to be subject to short lifetimes, low performance, and/or long startup times while others function well for several years (Benner et al., 1999, 2002; Blowes et al., 2000; Waybrant et al., 2002). Previous studies have attempted to address the issue of poor performance by focusing on the organic substrates used, and have evaluated straw (Bechard et al., 1994), sawdust (Wakao et al., 1979), peat (Eger and Lapakko, 1988), spent mushroom compost (Dvorak et al., 1992), whey (Christensen et al., 1996), and oak chips (Chang et al., 2000). However, no clear solution has been provided.

Although SR-PRZs are biologically catalyzed treatment systems, their microbial communities have not been well characterized. A few recent studies have investigated the kinds of microorganisms present in these systems using a combination of culture-based and molecular biological techniques (Benner et al., 2000; Hallberg and Johnson, 2005a; Johnson and Hallberg, 2003; Pereyra et al., 2005; Pruden et al., 2006). The results of these studies correlate well with the results of parallel research focusing on the carbon flow dynamics in these systems (Logan et al., 2005), and generally emphasize the complexity of the microbial communities responsible for remediation. Although SRB catalyze the final reaction of the SR-PRZ, they rely on the activity of anaerobic cellulolytic bacteria and fermentative bacteria to break down complex organic materials, such as cellulose from wood chips, to provide them with carbon and energy sources. Therefore, efforts to improve microbiological design criteria for SR-PRZs must consider the entire microbial community and not merely SRB.

The purpose of this study was to investigate the effect of inoculum on the performance of sulfate-reducing columns (SRC) simulating SR-PRZs. To gain a deeper understanding, denaturing gradient gel electrophoresis (DGGE) and real-time quantitative polymerase chain reaction (Q-PCR) were used to investigate the microbial community dynamics of the columns during startup and pseudo-steady-state operation. The results of this research provide insights for inoculum selection and may help improve the reliability of SR-PRZ performance in the field.

2. Materials and methods

2.1. Column specifications and packing

Six acrylic columns, 5 cm ID \times 15 cm height, with six $\frac{3}{4}$ -in vertical sampling ports, were supported vertically with upward flow at an average rate of 40 mL/d. Each column was packed under a nitrogen atmosphere with 100 g dry weight of a homogenized mixture consisting of 22% beech wood chips, 2% crushed alfalfa, 11% pine shavings, 45% silica sand, 5% limestone, and 15% inoculum by weight. This mixture is typical of SR-PRZs and includes slow-release carbon and nitrogen sources (woody material and alfalfa), a source of alkalinity (limestone), and an inert material for porosity enhancement (sand) (Waybrant et al., 1998, 2002). The bottom layer (1.9 cm) of all columns was packed with a 90/10 wt% mixture of silica sand/crushed pyrite to scavenge oxygen, as described by Waybrant et al. (2002). The top layer (0.6 cm) of all columns was packed with silica sand, separated from the organic layer by a stainless steel mesh to prevent organic material loss. All columns were covered in aluminum foil to inhibit photolithotrophic bacterial growth (Waybrant et al., 2002).

2.2. Inocula

Two inocula were compared in this study: (1) fresh bovine dairy manure (DM) collected from a dairy near Fort Collins, CO, and (2) contents from a previous column containing a sulfate-reducing community (SRC) (Logan et al., 2005). Two columns of each type (DM1, DM2, SRC1, SRC2) were prepared and compared to two uninoculated columns (U1, U2).

2.3. Influent composition

Columns were fed simulated mine drainage water from a common feed bottle. Due to problems with precipitation of iron in the influent bottle, the composition of the influent was variable during the initial 10 d of startup. During this time, the influent pH was adjusted from 6.0 to 5.6 and the influent Fe(II)SO₄ concentration was lowered from 0.49 to 0.034 g/L to reduce the extent of iron precipitation. The final influent composition was 1.32 g/L Na₂SO₄, 0.03 g/L NH₄Cl, 0.05 g/L ZnSO₄, 0.03 g/L Fe(II)SO₄, and 0.01 g/L CdCl₂ at a target pH of 5.6 (adjusted with HCl and NaOH as needed). Before adding the metals, the deionized water for the influent was bubbled with nitrogen for 2 d to remove dissolved oxygen. The final influent solution was continuously bubbled with a slow stream of nitrogen (10 mL/min) to replace the headspace as the influent was pumped to the six columns. No precipitate was noted in the influent bottle for the remainder of the experiment.

2.4. Column and effluent sampling

Effluent was collected from the top of each column in 250-mL flasks initially containing 25 mL of deionized water in which the column effluent tube was submerged to prevent entry of oxygen into the column. This initial volume was considered

in the calculation of sulfate and metal concentrations. Column material (approx. 0.5 g) was removed weekly from the middle sampling port of the DM and SRC columns for the first 6 weeks to monitor microbial community development. The uninoculated columns were sampled only at Week 6. Columns were disconnected from the flow system and manipulated under a nitrogen atmosphere during sampling.

2.5. Analytical methods

Sulfate was quantified using the turbidimetric SulfaVer[®] 4 method (Hach Co., Loveland, CO) with an Odyssey DR/2500 spectrophotometer (Hach Co.). After collection, aqueous samples were immediately filtered through 0.2- μ m syringe-tip filters and diluted with deionized water to achieve a concentration within the detection range of 2–70 mg/L. In order to verify that characteristics of the samples did not interfere with the turbidimetric method, samples were spiked with standard sulfate solutions. Based on these tests, no matrix effects were found.

Metals were analyzed by inductively coupled plasma absorbance emission spectroscopy (ICP-AES) (Thermo Jarrell Ash IRIS Advantage). Aqueous samples were filtered through 0.2- μ m syringe-tip filters, diluted with deionized water, and acidified with trace metals-grade nitric acid (Mallinckrodt, Hazelwood, MO). The acidified solution was then boiled down to one-tenth of the initial volume and diluted to 50 mL with deionized water for direct analysis by ICP-AES. Detection limits were 0.01 mg/L for iron and zinc, and 0.005 mg/L for cadmium. In every independent set of measurements, a deionized water blank was also measured. The calibration of the ICP-AES instrument was checked every 10 runs.

To determine the distribution of metals in the solid substrate of the columns at the end of operation, the column contents were divided approximately in thirds by volume into top, middle, and bottom sections. The material of these sections was well mixed and subsequently digested in a CEM microwave digester (MS-2000, Mathews, NC) per EPA Method SW-3015. The digested material was analyzed by ICP-AES as described above.

2.6. DNA extraction

DNA extractions were carried out using the FastDNA[®] Spin Kit for Soil (Q-BIOGene, Irvine, CA) according to the manufacturer's protocol using approximately 0.5 g of homogenized column material (the exact amount was recorded for quantification). Concentrations of DNA were determined spectrophotometrically (Hewlett Packard 8452A diode array spectrophotometer, Houston, TX) by measuring the absorbance at 260 nm. All DNA extracts were diluted 1:3 with sterile deionized water in preparation for polymerase chain reaction (PCR).

2.7. PCR amplification of 16S rDNA

A nested PCR approach was used to amplify the variable V3 region of the 16S rDNA molecule using primers 8F and 1492R (Weisburg et al., 1991) followed by I341F and I533R (Watanabe et al., 2001). The reaction (total volume 25 μ L) included 12.9 μ L

purified water, 2.5 μ L 10 \times Taq Reaction Buffer buffer, 5.0 μ L TaqMaster PCR Enhancer (Eppendorf, Hamburg, Germany), 5 pM of each dNTP, 0.25 pM of each primer, 0.25 μ L formamide, 1.75 U Taq DNA polymerase (Eppendorf), and 1 μ L DNA template. The temperature conditions for PCR were those described by Weisburg et al. (1991) for 8F and 1492R primers and by Watanabe et al. (2001) for I341F and I533R primers.

2.8. Denaturing gradient gel electrophoresis (DGGE)

Gels (8% acrylamide/bisacrylamide 19:1, BioRad) were cast using a denaturing gradient of 20–55%, with 100% denaturant defined as 7 M urea and 20% v/v formamide. A standard prepared with a mixture of DNA from pure cultures was loaded in all gels to verify the gradient. Gels were exposed to at 45 V for 20 h at 57.5 $^{\circ}$ C and stained with SybrGold nucleic acid stain (Molecular Probes, Inc., Eugene, OR). Gels were documented using a UVP BioChem gel documentation system and images were analyzed using Labworks software (UVP, Upland, CA). This software was used to identify the bands in the gels and to obtain the intensity profiles for each of the lanes. The intensity profiles were used to assess the degree of similarity between replicates at different times. The relative diversity was calculated using the Shannon diversity index:

$$H = - \sum \left[\frac{n_i}{N} \log \left(\frac{n_i}{N} \right) \right],$$

where n_i is the intensity of the individual bands and N is the sum of the intensity of all the bands (Cox, 1972; Xia et al., 2005). Representative visible DGGE bands (33 total) were excised with sterile razor blades and stored in 2-mL tubes with 36 μ L of sterile water.

2.9. DNA sequence analysis

DNA present in the DGGE bands was PCR amplified and purified for sequencing using the GeneClean Spin Kit (Q-BIOGene). Sequencing of the 200bp product was performed by Davis Sequencing (Davis, CA). The closest matches to known microorganisms available in the National Center for Biotechnology Information database were determined using the BLAST alignment tool (<http://www.ncbi.nlm.nih.gov/BLAST/>). A literature survey was conducted to characterize the properties of the closest matches with respect to substrate utilization and sulfate reduction.

2.10. Cloning

Eighteen of the 33 cut DGGE bands resulted in mixed sequences. To separate these, the excised bands were PCR amplified using primers I341F and I533R as described above, purified using the GeneClean[®] Spin Kit (Q-BIOGene), and cloned into *Escherichia coli* using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) according to the manufacturers' protocols. Inserts were PCR amplified directly from colonies using M13F and M13R primers. At least three clones per band were analyzed. PCR products were purified using the GeneClean[®] Spin Kit (Q-BIOGene) and sequenced by Davis Sequencing (Davis, CA).

2.11. Real-time Q-PCR

Q-PCR targeting the 16S rDNA of total bacteria and the SRB genus *Desulfobacterium* (DSB), which has commonly been identified in mine drainage systems (Hallberg and Johnson, 2005b; Morales et al., 2005), was performed using a Cepheid SmartCycler (Sunnyvale, CA). Primers were designed for the DSB subgroup in the δ -proteobacteria subdivision using FastPCR® software (Kalendar, 2005). The sequences of the DSB primers were AGTARAGTGGCGYACGGGTGAG for the forward primer (HDBM52f) and WTCAYYCACGCGGCG-TYGCTGC for the reverse primer (HDBM372r), yielding a product of approximately 320 bp. The specificity of the primers was verified both by a BLAST search and by shotgun cloning and sequencing of amplified products. Qiagen Sybr-Green MasterMix (Valencia, CA) was used to adapt the primers to Q-PCR. The template used for calibration was purified 16S rDNA PCR product obtained from *Desulfobacterium autotrophicum* ATCC43914D (American Type Culture Collection, Rockville, MD). Calibration curves were constructed using six points with four replicates for each point. In every Q-PCR run, three standards were included. The C_t values of these standards were compared to the corresponding values in the calibration curve, and if the difference was more than $\pm 5\%$, the data from that run were discarded. The limit of quantification of the DSB assay was 917 copies per reaction. For total bacteria, the conditions described by Suzuki et al. (2000) were used, including universal 16S rDNA primers and probe. A six-point calibration curve was generated using amplified 16S rDNA from a sample collected from a sulfate-reducing bioreactor as the template, with four replicates in two independent runs for each point. For both assays, a dilution series was performed on all samples and analyzed by Q-PCR to identify the linear range unaffected by inhibitors. A dilution of 1:20 was sufficient for most samples.

2.12. Statistical and mathematical analyses

Mixed linear regressions were fit to all data sets using the PROC MIXED function of SAS 9.1 (SAS Institute Inc., Cary, NC). Log transformation of all data sets was required to achieve homoscedasticity, and all comparisons were made in log scale. Degrees-of-freedom calculations were performed using the Kenward–Roger method with repeated time measurements. Significance was defined by a Type III p -value ≤ 0.05 . Dixon's Extreme Value test was used to test for statistical outliers.

Column pore volume was estimated using the ratio of pore volume to empty column volume based on Logan et al. (2005) as similar column materials and packing techniques were utilized in the present study. The pore volume was found to be 166 cm³ with a residence time of 4 d at an average flow rate of 40 mL/d.

For cumulative sulfate and metal removal analyses, cubic splines were fit to the effluent concentrations and flow rate data and then interpolated for direct comparison with the discrete influent data. Splines and interpolation were performed with the 'cspline' and 'interp' functions of MathCAD 12 (Mathsoft Engineering & Education, Inc., Cambridge, MA).

3. Results

3.1. Qualitative observations

Between the first and seventh weeks of operation, the color of the column material changed only slightly (from dark to light green) in the columns inoculated with DM (DM1, DM2), and there was some darkening (from light brown to gray) in the uninoculated columns (U1, U2). By contrast, the columns inoculated with preacclimated column substrate (SRC1, SRC2) changed markedly from light brown to black. Hydrogen sulfide odor was also detectable in the effluents of the SRC columns after 2 wk, whereas this odor was not apparent in the effluents of the other two sets of columns.

3.2. Cumulative sulfate removal

Cumulative sulfate removal (Fig. 1) was negligible in the DM and U columns until Day 30, while sulfate was removed at low rates in the SRC columns during this period. After this time, an increase in the rate of removal was observed in all columns, with the highest sustained rates occurring in the SRC columns. The DM and U columns maintained the higher removal rate for approximately 60 d, while the SRC columns maintained the rate for an additional 30 d (through Day 120). Significantly greater cumulative sulfate removal was observed in the SRC columns than in the DM ($p = 0.004$) and U columns ($p < 0.001$), and no statistical difference was determined between the DM and U columns ($p = 0.2731$).

3.3. pH

The effluent pH varied significantly with startup, followed by a gradual increase in all columns throughout the experiment. Between 40 and 125 d of operation, the pH of the SRC column effluent was consistently higher than that of the DM or U columns and fluctuated near pH 8 after Day 60. However, due to the fluctuation of the influent pH, no statistically valid distinction could be made among the columns. Because the influent was not buffered, a consistent pH in the influent was not attained (pH range 4–8, with an average and standard deviation of 5.61 ± 1.06). However, the buffering capacity was increased by the column contents, yielding a relatively stable effluent pH.

3.4. Cumulative metal removal

Calculated cumulative mass removals (Fig. 2) revealed that the total mass of cadmium and zinc removed by the SRC columns was greater than that removed by the DM ($p < 0.001$ for both metals) and U columns ($p < 0.001$ for both metals). No statistical difference in cumulative cadmium removal was observed between the U and DM columns ($p = 0.552$), and cumulative zinc removal in the DM columns was statistically greater than the removal in the U columns ($p = 0.002$). For the duration of the experiment, the rate of removal for both cadmium and zinc was greater in the SRC columns than in the DM and U columns. In the case of iron (data not shown), cumulative removal in the SRC and U columns was

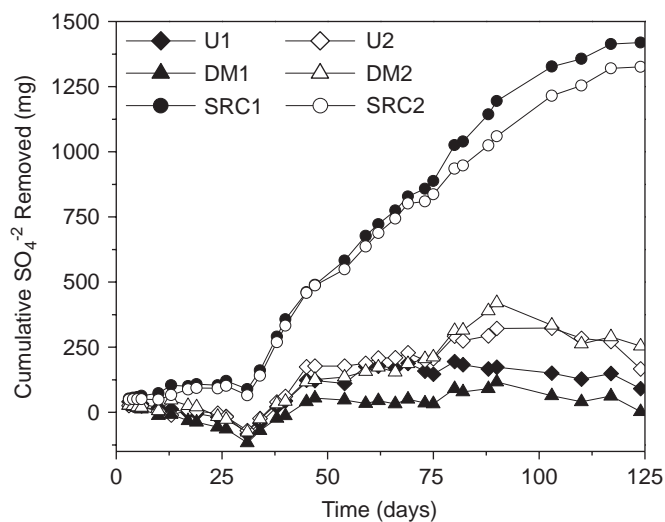


Fig. 1 – Cumulative sulfate removal in duplicate SR-PRZ columns that were uninoculated (U) or inoculated with an acclimated culture (SRC) or with bovine dairy manure (DM). Cubic splines were fit to the effluent sulfate concentrations and flow rate data and then interpolated for direct comparison with the discrete influent data using a residence time of 4 d. The negative cumulative removal values for the DM and U columns during the first 30 d can be attributed to the variable influent concentrations at the beginning of the experiment and to the propagation of error associated with the mathematical treatment of the minimal sulfate removal during this time.

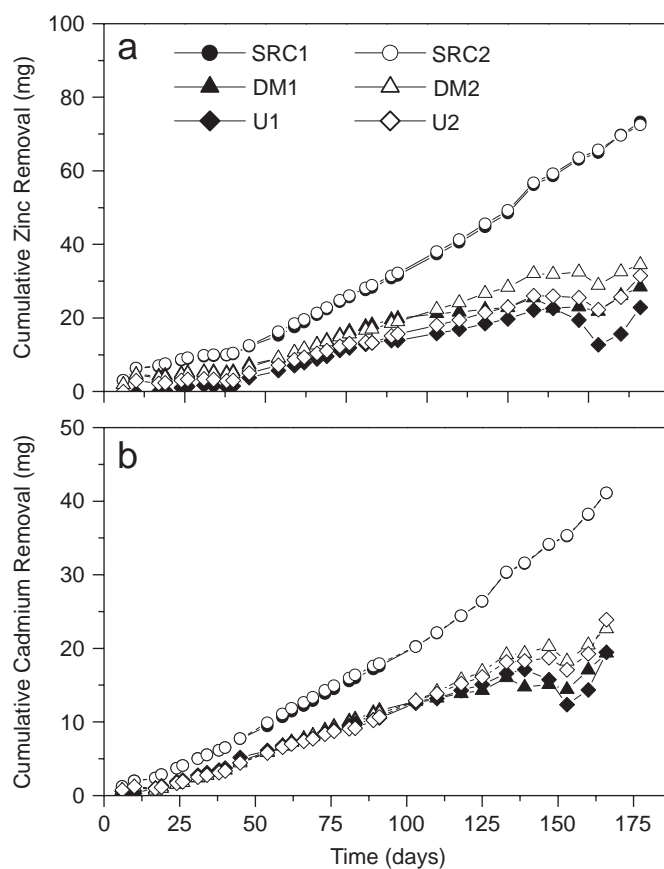


Fig. 2 – Cumulative zinc (a) and cadmium (b) removal in the uninoculated columns (U) and columns inoculated with an acclimated inoculum (SRC) or with bovine dairy manure (DM). Cubic splines were fit to the effluent metal concentrations and flow rate data and then interpolated for direct comparison with the discrete influent data using a residence time of 4 d.

significantly higher than in the DM columns ($p < 0.001$ for both column sets) but no significant differences in removal were observed between SRC and U columns ($p = 0.972$).

3.5. Distribution of metals within the columns

In the solids of the DM1 and U2 columns, the cadmium and zinc concentrations did not differ between the top, middle, and bottom ($p > 0.1$) (Fig. 3). In the DM2 columns, the concentrations of these metals were slightly lower in the bottom (inlet) portion. In the SRC columns, concentrations of cadmium and zinc were lower in the upper section than in the middle and bottom. However, in the SRC1 column, most of the cadmium and zinc was near the inlet, whereas these metals were equally concentrated in the bottom and middle of the SRC2 column.

Iron concentrations were highest in the inlet section of the DM and U columns (data not shown). In the SRC columns, however, the iron concentrations were more equally distributed among the three portions of the columns, with the highest concentration in the middle (SRC2) or in the bottom (SRC1).

3.6. DGGE of column microbial communities

Using the criterion that a microbial community reached a pseudo-steady state when no new DGGE bands were detected

and no existing bands disappeared, the microorganisms in the SRC columns formed a stable community (after Week 2) before the other columns (Fig. 4a). By contrast, the DM columns still had new detectable bands at Week 4, while three bands faded gradually after this time in the DM1 column (Fig. 4b). More bands were observed in the DGGE gels for SRC columns than in the gels for DM or U columns. However, the Shannon diversity indices for the SRC and DM columns were not significantly different ($p = 0.136$). Diversity indices for the two SRC columns were highly similar and ranged from a value of $H = 1$ at the beginning of the experiment to $H = 1.37$ at Week 6. Diversity indices for column DM2 were consistently lower than those for column DM1 until Week 6, at which time the index values for both columns converged. Diversity index for column DM2 increased from an initial value of $H = 0.5$ to a value of $H = 1.12$ at Week 6. The initial average diversity index in column DM1 was $H = 1.26$. This index decreased to an average value of $H = 1.08$ by Week 1 and remained constant at all other sampling points.

Table 1 summarizes the results obtained by sequencing DGGE bands. Of the 30 unique bands of the SRC columns, 15 were sequenced, while eight of 27 unique bands were sequenced from the DM columns. Several cellulose-degrading bacteria belonging to the *Eubacterium*, *Clostridium*, and *Bacteroides* genera were found in the SRC columns. Fermentative bacteria in SRC columns were related to the classes

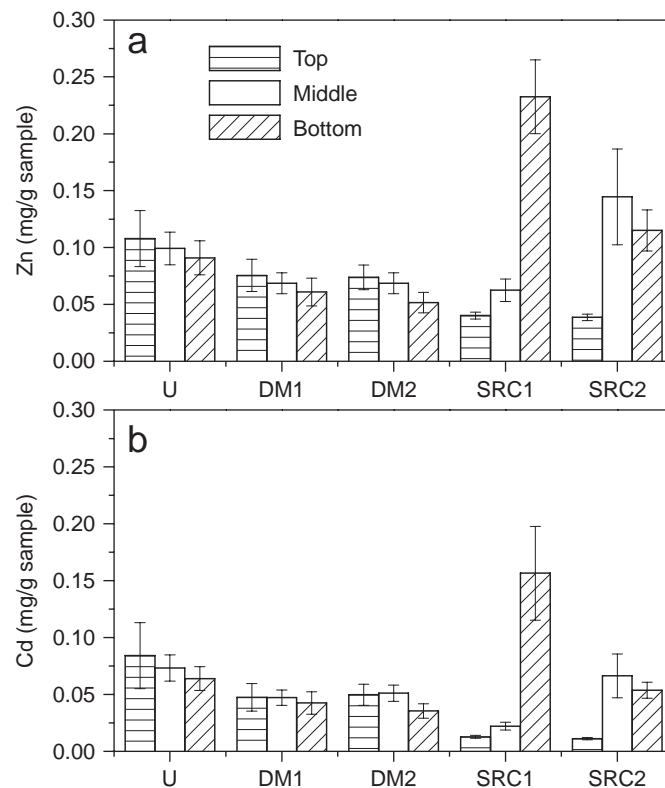


Fig. 3 – Distribution of (a) cadmium and (b) zinc in the columns determined by ICP-AES analysis of digested column material collected from the top, middle, and bottom sections of each column after 170 d of operation. The influent entered the columns from the bottom. Results for only one uninoculated column are shown because the second was used for a tracer test. Error bars represent standard deviations of the means of five replicates.

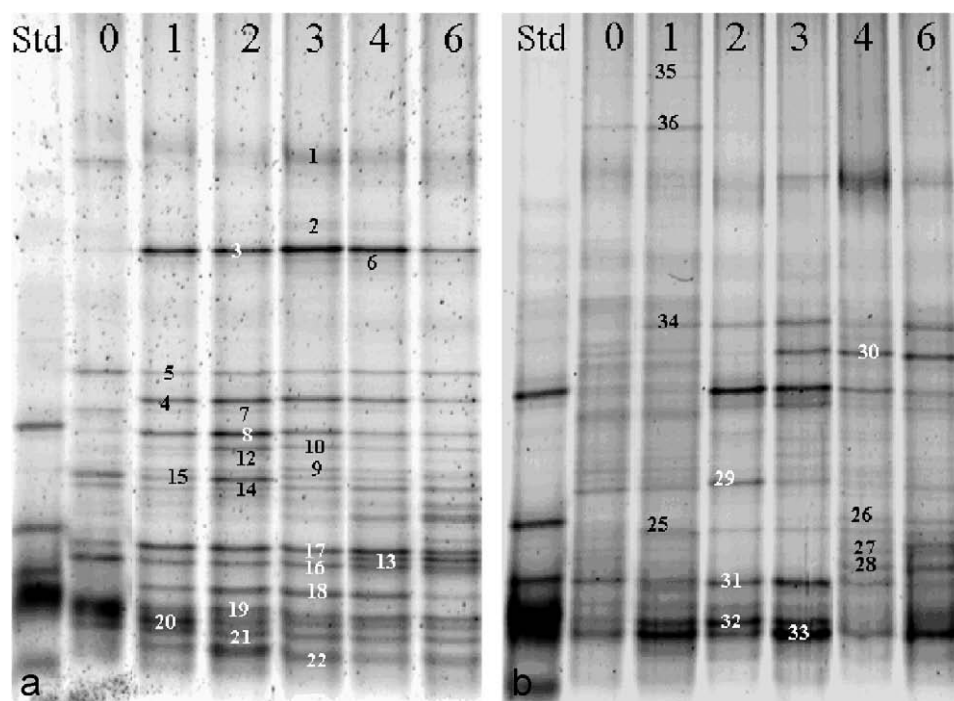


Fig. 4 – DGGE gel image of nested I341F and I533R PCR products of DNA extracted from (a) column SRC1 and (b) column DM1 during the first 6 wk. Lane numbers correspond to the sampling week. ‘std’ is the positive control. Twenty-three of the labeled bands were sequenced and their identities are indicated in Table 1.

Enterobacteria, Clostridia, and Bacteroidetes. In DM columns, the majority of the fermenters and the one cellulose degrader identified were related to members of the genus *Clostridium*. An SRB was identified in the SRC columns, and an SRB was also detected in one of the U columns (data not shown). No SRB were detected in the eight sequenced DGGE bands from the DM columns.

3.7. Q-PCR

Although the total bacterial population in the DM columns was initially larger than that in the SRC columns, it rapidly decreased during the first 3 wk and was similar to the SRC columns for the remainder of the experiment (data not shown). At Week 6, the final sampling point, the bacterial populations of DM and SRC columns were not significantly different in size from those of the U columns.

Overall, the DSB populations in the SRC columns were significantly larger than in the DM columns ($p = 0.027$) (Fig. 5a). The number of DSB 16S rRNA gene copies increased in the SRC columns at Week 2 while it decreased in the DM columns. At Week 6, the DSB populations in the U columns were significantly smaller than those in the SRC ($p = 0.021$) and the DM ($p = 0.010$) columns.

The DSB population also constituted a much larger fraction of the bacterial community in the SRC columns than in the DM and U columns (Fig. 5b). At the beginning of the experiment, the ratio of DSB to total bacteria was smaller in the DM columns ($p = 0.022$). By Week 2, this ratio had increased in the SRC columns while it did not change in the DM columns. The ratios of DSB to bacteria in the SRC and DM columns were significantly different

($p < 0.001$). The higher ratios for the SRC columns are in agreement with the superior metal and sulfate removal observed for these columns.

4. Discussion

4.1. Effect of inoculum on column performance

Metal removal, pH neutralization, and sulfate removal can be used as indicators of column performance. This work indicates that the microbial inoculum significantly affects the performance of columns remediating acid mine drainage (AMD). The rates of removal of sulfate and metals in the SRC columns were superior to those in the DM and U columns (except in the case of iron, which was removed by both the SRC and U columns better than by the DM columns). Interestingly, the columns inoculated with DM, a common source of inoculum (Castro et al., 1999; Christensen et al., 1996), did not perform better than the uninoculated columns. This suggests that the ‘wrong’ inoculum may not provide an advantage over no inoculum at all.

In addition to remediation performance, the startup time and duration of active remediation (pseudo-steady state) are important parameters in determining the overall column performance. For comparison, startup time was taken as the point when sulfate or metal removal was observed, independent of removal rate, and pseudo-steady state was considered as the time during which the rate of removal was constant. Cadmium and zinc removal was observed in all columns from the start of the study, indicating an immediate startup to a pseudo-steady state. The pseudo-steady state was

Table 1 – Characterization of microorganisms represented by DGGE bands

Band ^a	Highest match (GenBank accession number)	% Match	A ^b	B ^b	C ^b	D ^b	Ref.
SRC							
1	<i>Clostridium indolis</i> strain DSM 755(Y18184)	99	–	+	+	–	(Dworkin, 2000; Holt, 1984; Leschine, 1995; Palop et al., 1989)
	<i>Clostridium celerecrescens</i> strain EIB 5 (AY458859)	99	+	–	+	–	
2	Uncultured bacterium BTCE-T2 1F 16S ribosomal RNA gene (AY217446)	96					
3	<i>Clostridium fimetarium</i> (AF126687)	99	–	–	+	–	(Kotsyurbenko et al., 1995)
4	<i>Eubacterium cellulosolvens</i> strain Ce2 (AY178842)	100	+	+	+	–	(Bryant et al., 1958)
6	<i>Bacteroides</i> sp. 253c (AY082449)	94	+	+	+	–	(Dworkin, 2000)
7	<i>Desulfovibrio vulgaris</i> strain I5 (AY362360)	96	–	–	–	+	
8	<i>Clostridium longisporum</i> strain DSM 8431	98	+	+	+	–	(Varel, 1989)
10	Uncultured bacterium clone BCf5-21 (AB062828) that was 92% similar to an uncultured <i>Clostridiaceae</i> bacterium (AB089033)	91					
12	<i>Klebsiella pneumoniae</i> (AY369139)	92	–	–	+	–	(Dworkin, 2000)
13	Uncultured isopod gut bacterium clone RKP5AM (AF395327) that was 93.6% similar to an unidentified <i>Bacteroides</i> spp. (AF150715)	88	+	+	+	–	
14	Uncultured <i>Clostridiaceae</i> bacterium clone: Rs-Q69 (AB089029)	96			+		(Dworkin, 2000)
16	<i>Clostridium longisporum</i> strain DSM 8431 (X76164)	99	+	+	+	–	(Varel, 1989)
20	<i>Escherichia vulneris</i> (AF530476)	87	–	–	+	–	(Brenner et al., 1982)
21	<i>Clostridium longisporum</i> strain DSM 8431 (X76164)	99	+	+	+	–	(Varel, 1989)
22	<i>Escherichia senegalensis</i> (AY217654)	93	–	–	+	–	(Dworkin, 2000)
DM							
25	Uncultured bacterium RSa32 (AJ289204). Related to <i>Clostridium polysaccharolyticum</i> (X77839)	99	+	+	+	–	(Holt, 1984; Vangylswyk, 1980)
26	Uncultured bacterium clone HuCB56 (AJ409006) that was 94% similar to <i>Eubacterium xylanophilum</i> (L34628),	99	–	+	+	–	(Vangylswyk and Vandertoorn, 1985)
27	<i>Clostridiaceae</i> bacterium 80Kb (AB084627) that was 91.5% similar to <i>Clostridium leptum</i> (AF262239)	95	–	–	+	–	(Holt, 1984; Moore et al., 1976)
28	<i>Sporomusa malonica</i> strain DSM 5090 (AJ279799)	81	–	–	+	–	(Dehning et al., 1989)
29	<i>Clostridium indolis</i> strain DSM 755 (Y18184)	95	–	+	+	–	(Dworkin, 2000; Holt, 1984)
32	Uncultured bacterium clone Thompsons76 (AY854354)	88					
34	<i>Clostridium butyricum</i> strain EIB 3-4 (AY458857)	94	–	+	+	–	(Dworkin, 2000; Holt, 1984)
35	<i>Clostridium lituseburense</i> strain EIB 6 (AY458860)	82	–	–	+	–	(Holt, 1984)

^a Band numbers correspond to numbering of Fig. 4.

^b A: cellulose degradation; B: fermentation of polysaccharides; C: fermentation of monosaccharides (and/or disaccharides); D: sulfate reduction. Where more than one sequence had the same similarity with the DGGE sequence, all the results corresponding to the highest matches are presented. When the DGGE band sequence corresponded to an uncultured bacterium the function of the microorganism was assigned based on the functions of the closest known relative.

maintained throughout the experiment in the SRC columns, while the DM and U columns displayed a decline in removal rate after Days 125 and 133 for zinc and cadmium, respectively, indicating the end of the pseudo-steady state period. The startup was also immediate in terms of sulfate removal in the SRC columns, but required more than 4 wk for the DM and U columns. Pseudo-steady state did not begin until Day 30 for all columns. The active remediation lasted for 90 d (through Day 120) in the SRC columns at a significantly higher removal rate than the DM and U columns, which remained in pseudo-steady state for only 60 d (through Day 90). These results suggest that the type of inoculum does not affect the startup time for metal removal (which may have been achieved partly by sorption in the first several days); however, inoculation with a preacclimated culture resulted in a shorter startup time for sulfate removal and maintenance of remediation capabilities for a longer period than the use of DM or

no inoculum. A longer experiment would be required to fully simulate operation times in the field, which vary from 2 to 20 years (Benner et al., 1999, 2002; Blowes et al., 2000; Waybrant et al., 2002).

These findings agree with results of other studies that show an effect of microbial inoculum on system performance. Moreno et al. (2005a) found that inoculating a submerged filter for biological denitrification with a pure culture of denitrifying bacteria increased system stability and performance indicators compared to inoculation with a mixed culture from a wastewater treatment plant's activated sludge. In the same system, Moreno et al. (2005b) also showed that the choice of microbial inoculum can also influence startup time. Pereira et al. (2001) verified that the degradation of oleic acid in anaerobic filters was improved by inoculating the system with acclimated biomass compared to nonacclimated biomass.

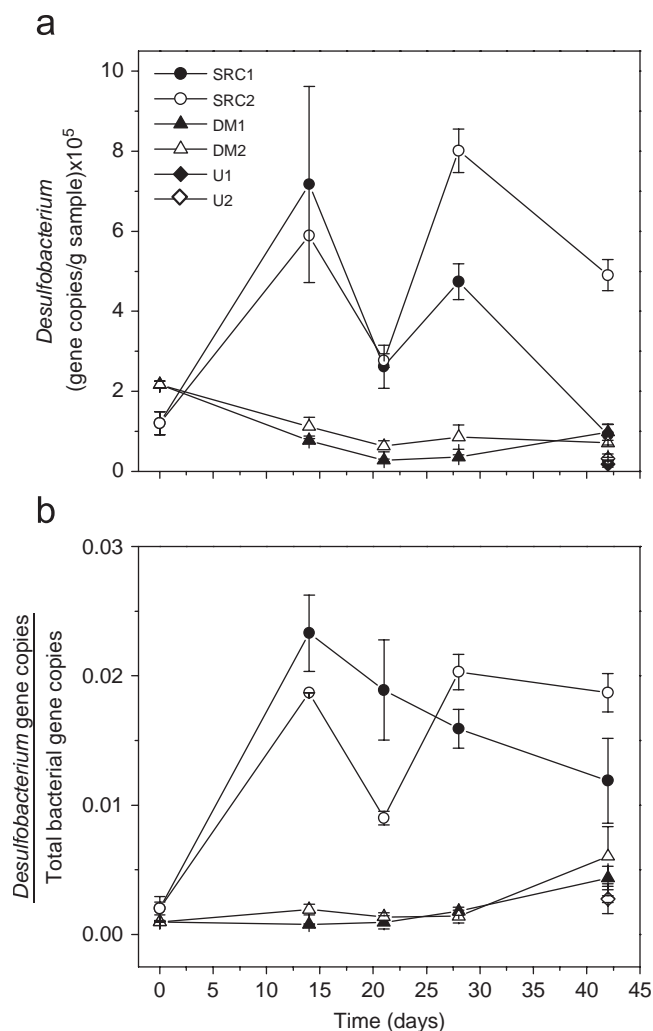


Fig. 5 – Quantification of *Desulfobacterium* with time by Q-PCR for SRC, DM, and U columns (a) normalized to mass of sample used for DNA extraction and (b) normalized to total bacteria. Error bars represent standard deviations of the means of two to six replicates.

4.2. Spatial distribution of metal removal

Analysis of cadmium, zinc, and iron in the column material revealed spatial heterogeneity in the SRC columns (Fig. 3). In particular, zinc and cadmium concentrations in the bottom and middle portions of the SRC columns may have been higher because the higher levels of sulfate reduction in the SRC columns precipitated the metals more efficiently near the inlet, preventing them from reaching the upper layers at high concentration.

4.3. Microbial community composition and dynamics

Microbial community profiling by DGGE provided data to determine the time required to reach a pseudo-stable microbial community, to identify microorganisms, and to compare the relative diversity. Based on DGGE, the microbial

communities in the SRC columns achieved a stable composition sooner than the populations in the DM and U columns. This correlates well with the performance data, and is a promising suggestion that the composition of the microbial inoculum can influence the startup time of a SR-PRZ, an issue that is significant for field operation. In a bench-scale study of AMD treatment using microbial-driven sulfate reduction, Christensen et al. (1996) demonstrated that inoculating the system with SRB shortened the initial lag phase.

The majority of the bands sequenced from the SRC columns corresponded to microorganisms that degrade cellulose and other polysaccharides, such as pectin and starch, and ferment the products of the hydrolysis (e.g., *Clostridium longisporum* and *Eubacterium cellulosolvens*). Sequences related to *Bacteroides* spp. were found only in SRC columns. *Bacteroides* spp. are saccharolytic microorganisms and, as a group, are able to utilize a wide variety of compounds as carbon and energy sources, including cellulose, hemicellulose, starch, and pectin (Dworkin, 2000). The mechanisms of polysaccharide digestion in *Bacteroides* spp. are highly effective (Dworkin, 2000). By contrast, only one of the sequences from the DM columns (DGGE Band 25) was confirmed to be related to a cellulose degrader. In the DM columns, 88% of the sequenced bands corresponded to microorganisms that ferment di- and/or monosaccharides and 57% of these microorganisms were also able to degrade polysaccharides such as starch and pectin. Since the degradation of cellulose has been shown to be the rate-limiting step in the carbon flow dynamics in SR-PRZs of the type tested here (Logan et al., 2005), the presence of *Bacteroides* spp. together with a diverse population of additional polysaccharide fermenters in the SRC columns might have constituted an advantage of these columns over the DM columns.

Although sulfate was removed by the DM columns, no SRB were found in the sequenced DGGE bands. In the SRC columns, the sequence from Band 7 showed 96% similarity to the sulfate reducer *Desulfovibrio vulgaris* strain 15. This band was detected by DGGE in Week 2. Both *Desulfovibrio* spp. and DSB spp., which were quantified by Q-PCR, have been identified as major SRB in AMD treatment systems (Hallberg and Johnson, 2005a; Labrenz and Banfield, 2004). SRB have been observed to represent a relatively low proportion of the microbial community in other sulfate-reducing mine drainage treatment systems (Hallberg and Johnson, 2005b; Johnson and Hallberg, 2003; Morales et al., 2005). This may explain why more SRB were not detected by DGGE and is corroborated by the low proportion of DSB (0.1–2%) detected by Q-PCR in all of the columns. This also supports the hypothesis of the importance of the upstream microorganisms that provide SRB with carbon substrates for growth (cellulose degraders and fermenters). A higher proportion of SRB in the SRC columns together with a variety of microorganisms capable of degrading complex organic materials might have caused the increased metal removal and pH neutralization observed in these columns compared to the DM columns.

Q-PCR analysis also revealed that the DM columns initially contained more total bacteria than the SRC columns. The decrease in total bacteria in DM columns in the following weeks could be an indication that the conditions were not

favorable for a large portion of the original microbial community. This is not surprising considering the stark contrast in environmental conditions between the columns and the thermophilic lower intestinal tract of a dairy cow. It is also possible that the nutrient availability at the beginning of the experiment (especially the presence of readily available soluble organics) caused competition of different microbial groups that ultimately lead to the survival of only those that were better at procuring these nutrients, but not necessarily better at the activities that lead to heavy metal precipitation.

5. Conclusions

The results of this study demonstrate that the nature of the inoculum influences the performance of sulfate-reducing systems treating heavy metal contaminated water. An inoculum previously acclimated to sulfate-reducing conditions and complex organic substrates outperformed DM, a typical SR-PRZ inoculum. The application of biomolecular tools provided deeper insights into the composition and overall dynamics of the microbial communities in the columns. The results of this study provide justification and basic information needed to support future efforts in the development of SR-PRZ inocula for improving reliability and performance in the field.

Acknowledgments

The authors thank EPA STAR Grant number R8395101-0 for funding this project and the Hazardous Substance Research Center at Colorado State University. Financial assistance was also provided by the National Science Foundation Research Experience for Undergraduates program in Water Research at Colorado State University. The authors would like to thank Ruoting Pei, HyunSuk Hong, Carmen Sans, and Santiago Esplugas for their help in performing effluent tests and substrate extractions, and Tom Sale and Dave Gilbert for providing assistance in column design and laboratory space for experiments.

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